ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2012) **10**(1); 5–13
doi:10.1017/S147926211100089X

Conservation of genetic diversity in regenerated landraces of Italian ryegrass

J. E. López[1] and J. A. Oliveira[2]*

[1]*Centro de Investigacións Agrarias de Mabegondo, Instituto Galego de Calidade Alimentaria, Consellería do Medio Rural, Xunta de Galicia, Apdo. 10, 15080 A Coruña, Spain and* [2]*Área de Producción Vegetal, Departamento de Biología de Organismos y Sistemas, Escuela Politécnica de Mieres, Universidad de Oviedo, 33600 Mieres, Spain*

Received 27 July 2011; Accepted 27 October 2011 – First published online 28 November 2011

Abstract

The objective of this study was to investigate the effect of one cycle of seed regeneration on the conservation of genetic diversity in five Italian ryegrass landraces (*Lolium multiflorum* Lam.). Regeneration took place outdoors, in a sheltered site surrounded by tall Galician wheat, 20 m from the nearest source of alien pollen. A balanced mixture of seed (the same weight of seed per plant) was made from 90–100 plants harvested within each population. The conservation of allele frequencies was assessed by starch gel electrophoresis. Five enzyme systems from 78–153 plants per population were examined on slices of a single histidine–citrate starch gel. Each regenerated population differed from its original landrace in at least one of the five loci. The mean heterozygosity per locus was 0.45 for original and regenerated populations, and the mean number of alleles per locus was 3.7 and 3.6 for original and regenerated populations, respectively. There was no loss of common alleles (frequency >0.05) in the five regenerated populations compared with the original populations. Only three rare alleles (frequency <0.05) were lost (e.g. alleles phosphoglucose isomerase (PGI)-2a, PGI-2c* and shikimate dehydrogenase (SDH)-1d in Padrón, Pravia and Luarca, respectively). No regeneration effect ($P > 0.05$) was observed in the six agromorphological characters. However, a significant landrace effect was observed ($P < 0.05$) in the five agromorphological traits and the regenerated landraces deviated from the original landraces in 20% of direct comparisons. The results suggest that the method of regeneration used was not very suitable for maintaining the genetic integrity of the original landraces.

Keywords: genetic resources; germplasm regeneration; *Lolium multiflorum* Lam

Introduction

Intensive production systems based on two crops per year, including fodder maize and Italian ryegrass, are used in small exploitations in the humid region of northwest Spain (Lloveras, 1987). Italian ryegrass occupies the land for about 6 months, between sowing in autumn and harvesting in spring, before maize is sown (Piñeiro and Pérez, 1992).

The success of the crop is based on its high production during winter, derived from the early ripening of local varieties (Piñeiro and Pérez, 1986).

The Centre for Agrarian Research (CIAM), which pertains to the Ministry of the Rural Environment of the Xunta de Galicia, maintains a collection of 1300 accessions of temperate forage grasses and legumes from northern Spain. Most of these accessions display a high degree of agromorphological and isoenzymatic variability (López *et al.*, 2011).

Interest in these local varieties of annual Italian ryegrass has led to the agronomic and isoenzymatic characterization of the material, with the aim of conserving

* Corresponding author. E-mail: oliveira@uniovi.es

diversity among local accessions of the species and evaluating their use in genetic improvement programmes (Oliveira *et al*., 1997). This has resulted in the recent inscription in the Catalogue of Spanish varieties (Orden APA/660/2003 de 12 de marzo, BOE de 25 de marzo de 2003) of the 'Pomba' cultivar, obtained by selection from a local population of annual Italian ryegrass in the area of Padrón (A Coruña, Galicia, NW Spain).

Regeneration of the accessions is required in order to produce enough seed to carry out field trials and to restore germination ability, which decreases after the storage of seed for several years (Soengas *et al*., 2009). Regeneration procedures should preserve the genetic integrity of the collection (Sackville Hamilton and Chorlton, 1997).

Anemophilic, allogamous and autoincompatible species (Fearon *et al*., 1983) such as Italian ryegrass display high genetic variance within populations, a high potential for genetic changes by genetic drift and selection during regeneration, and present a high risk for cross-pollination between regeneration plots if they are not adequately isolated (Sackville Hamilton and Chorlton, 1997). In these species, the need for isolation limits the number of accessions that can be regenerated in the same area (Guy *et al*., 1989).

The magnitude of genetic drift (random change in allele frequencies) depends on the effective population size, which is determined by factors such as the number of individuals contributing equally to the next generation (Breese, 1989). Because of large differences in the seed yield per plant within each accession, collection of equal amounts of seed from each plant is usually recommended to prevent genetic changes and possible loss of adaptive alleles (Breese and Tyler, 1981). This method involves intense effort and is therefore not always used, especially when resources are limited. In general, the seed is collected at the same time from all plants of each accession. The effects of genetic drift would become more pronounced with numerous regeneration cycles (Bradley and Johnson, 1997).

In contrast to drift, which affects all polymorphic loci, selection only affects traits (non-neutral loci) for which there is genetic variation associated with differential survival or reproduction in the regeneration environment. Regenerating accessions in a common environment is likely to impose convergent selection pressure, thus reducing diversity among accessions (Sackville Hamilton and Chorlton, 1997).

The gene flow between accessions during seed regeneration occurs if the accessions are not fully isolated from accessions of the same species, also reducing diversity among accessions (Johnson *et al*., 1996).

A study of the causes and effects of genetic variation within and between populations is essential in population genetics. Although isozyme studies have largely been replaced by other approaches based on DNA analysis (such as direct DNA sequencing, single nucleotide polymorphisms and microsatellites), they remain the fastest, cheapest marker systems, and are an excellent choice for projects in which identification of only low levels of genetic variation is required (Medina *et al*., 2005; Rodríguez *et al*., 2009). Virk *et al* (2000) compared the discriminatory capacity of four methods of identifying varieties of rice (isozyme analysis, amplified fragment length polymorphism (AFLP), microsatellite and inter-simple sequence repeat (ISSR)), and reported that except for ISSR, the other techniques are suitable for categorizing varieties of rice, with AFLP and isozyme analysis being the most efficient. AFLP markers have a high discriminatory power because they generate several bands that can be analysed in a single gel. However, these techniques include more stages and require DNA of a high level of purity, take longer to carry out and are relatively expensive (Lee and Henry, 2001).

In the case of allogamous species with anemophilous pollination, there is little information about how the allele frequencies and agronomic characteristics of populations are affected by the method of regeneration used in the germplasm banks. Balfourier *et al.* (1994) used isoenzyme analysis to study how the method of creating experimental populations affects the conservation of allele and genotypic frequencies in natural populations of perennial ryegrass (*Lolium perenne* L.). Johnson (1998) also used isoenzyme analysis to investigate the genetic structure of populations of Italian ryegrass regenerated in different ways.

The objective of the present study was to evaluate possible changes in genetic diversity by the use of isoenzymatic markers and morpho-agronomic traits in a first cycle of seed regeneration, by collecting balanced mixtures (equal weight of seeds per plant) of the seed within each population of local varieties of annual Italian ryegrass.

Materials and methods

Five Italian ryegrass landraces were chosen from two agronomic classes distinguished by the application of multivariate methods to the agronomic characterization of landraces of Italian ryegrass carried out by Oliveira *et al.* (1997). The landraces selected from agronomic group 1 were Luarca, Pravia and Prendes, with an intermediate flowering date (late March to early April). The landraces selected from agronomic group 2 were Ordes and Padrón, with a later flowering date (second half of April).

With the aim of obtaining seeds in the autumn of 2008, the five original landraces were sown under greenhouse conditions and transplanted to the field in the winter of

https://doi.org/10.1017/S147926211100089X Published online by Cambridge University Press

2007 (100 plants per landrace). The soil was covered with black plastic mesh to prevent the growth of weeds.

Since Italian ryegrass is an allogamous species, regeneration plots were isolated by a crop of autochthonous Galician wheat, some 20 m from any source of contaminating pollen (another source of propagation, cultivation, natural populations, etc.). The plants were cut once to delay the ripening date to the period when the wheat was sufficiently high. At the end of the flowering period, the flower spikes from each plant were placed in separate fibre bags to collect the seeds. The seeds were then processed, and maintained in cold storage under controlled conditions of humidity and temperature.

In the present study, seeds were collected from 90 to 100 plants of each landrace, and a balanced mixture was produced, i.e. the same weight of seed was included per plant, so that each genotype contributed equally (with respect to weight) to the total mixture.

During the winter of 2008–2009, seeds from the ten landraces (i.e. the five original and the five regenerated landraces) were sown under greenhouse conditions along with the commercial variety 'Vitesse'. Plants were transplanted to field trials after 2 months of growth in a randomized complete block trial with five replicates, using ten plants per landrace and replicate. There were therefore a total of 10 plants × 5 replicates × 10 landraces (5 original + 5 regenerated) = 500 isolated plants, and (10 plants × 5 replicates) = 50 plants of the commercial variety 'Vitesse'. Individual plants were transplanted at a distance of 0.5 m between lines and 0.5 m between plants. Fertilizer (NPK, 8:15:15) was applied at a rate of 1000 kg/ha. The field trial was carried out at the CIAM (43°15′N, 8°18′W) in A Coruña (Spain); plots were situated at an altitude of 100 m, close to the coast. Italian ryegrass is an annual species, so that the entire experiment was repeated in the following year (2010).

The following agromorphological traits were observed during 2 years:

hed: time of inflorescence emergence (number of days from 1 January).

dmy: total annual dry matter yield (g dry matter yield per individual plant).

alt: plant height (total height in cm) at the time of inflorescence emergence.

ain: number of inflorescences (number of spikes per individual plant) at the end of inflorescence emergence.

flw: maximum width of the flag leaf (mm), at the time of inflorescence emergence.

fll: maximum length of the flag leaf (cm), at the time of inflorescence emergence.

The regeneration effect was evaluated by a two-factor analysis of variance (ANOVA; landrace with ten levels, i.e. the five original landraces and the five regenerated landraces, and regeneration with two levels, no regeneration and regeneration), applied independently to the data from each year and for each variable. The ANOVA model used was as follows:

$$X_{ijkl} = \mu + b_i + L_j + R_k + E_{ijkl},$$

where X_{ijkl} is the phenotypic value of the considered trait; μ is the overall mean; b_i is the replicate effect; L_j is the landrace factor; R_k is the regeneration factor; E_{ijkl} is the error. The replicate effect was considered as random and the landrace and regeneration effects as fixed. Before the analysis, the normality of the residuals was tested by the Shapiro–Wilk (W) test, and the homogeneity of residual variances was tested by Levene's test. Separation of landrace means was performed by Duncan's test. All statistical tests were performed with the SAS statistical package (SAS Institute, 1999).

For isozyme analysis, between 78 and 153 plants from the ten original and regenerated landraces were used. The seeds were sown in trays and maintained under greenhouse conditions. Plants were analysed when they had four well-formed leaves. Standard techniques of starch gel electrophoresis were applied according to Hayward *et al.* (1995). Histidine–citrate buffer used was for five enzyme systems: phosphoglucose isomerase (PGI-2, *EC* 5.3.1.9.); phosphoglucose mutase (PGM-1, *EC* 2.7.5.1.); acid phosphatase (ACP-1, *EC* 3.1.3.2); shikimate dehydrogenase (SDH-1, *EC* 1.1.1.25); peroxidase (PEX-1, *EC* 1.11.1.7). The allelic nomenclature followed that of Hayward *et al.* (1995), where allele a^+ migrates faster than allele a, and c^* is intermediate between alleles c and d. Leaves were crushed in cold conditions (0–2°C). The extraction solution used was 0.7 M Tris–HCl (pH 7.2) and 1% mercaptoethanol. The resulting extract was absorbed with Whatman filter paper (number 3) placed on Bio-Rad medium containing starch gel. The genetic marker was *Lolium temulentum*, because it is homozygous for all systems (lines 1 and 15 of 30 in total gel). Histidine–citrate was used as a migration buffer. The gels, prepared with 30 migration lines, were subjected to stresses of 300–320 V for 18 h and then cut into slices of about 2 mm thick. Allele frequencies were determined by direct counting or by saving a digital picture for later interpretation. Standard statistics were calculated in order to characterize the genetic variability in populations, with the BIOSYS 1 program (Swofford and Selander, 1981). Allele distribution can be classified according to Marshall and Brown (1975) by allele frequency of common alleles (≥ 0.05) and rare alleles (< 0.05), and by allele occurrence in widespread alleles when occurring in $\geq 25\%$ of populations; localized alleles therefore occur in only one or in $< 25\%$ of

https://doi.org/10.1017/S147926211100089X Published online by Cambridge University Press

populations. Given that only five Italian ryegrass populations were sampled, any allele occurring in two or more populations will be considered as a widespread allele; otherwise it will be a localized allele. For each population and locus, the χ^2 test (Snedecor and Cochran, 1967) was used to compare the allele proportions in the original populations with those in the regenerated populations. The following statistics were calculated: mean number of alleles per locus (*A*); mean heterozygosity expected under panmixia (He); Wright's fixation indices or *F*-statistics (Wright, 1965). *F*-statistics describe the level of heterozygosity in a population; more specifically, the degree of a reduction in heterozygosity when compared with Hardy–Weinberg expectations. Thus, F_{IT} represents the deficit of heterozygotes relative to the total population (ten landraces combined), whereas F_{IS} represents the relative deficit of heterozygotes for each subpopulation (averaged for the ten landraces). A zero value of F_{IS} implies that no forces (drift, selection, assortative mating, inbreeding, etc.) are acting on each subpopulation, neither to bring the heterozygosity frequency up nor down. F_{ST} is the fixation index representing the differentiation level of the populations. It reflects to what degree the populations are subdivided (Hartl and Clark, 1997).

Results

No regeneration effect ($P > 0.05$) was observed for any of the six agromorphological variables in either of the 2 years. However, the landrace effect was significant ($P < 0.05$) for five (hed, dmy, alt, ain and fll) of the six agromorphological traits in both years. This is because the landraces were chosen from two different agromorphological groups. Agromorphological traits of the regenerated landraces differed significantly from their original landraces in three of 18 instances (6 agromorphological traits × 3 landraces) in the landraces in agronomic group 1 (Tables 1 and 2) and in three of 12 instances (6 agromorphological traits × 2 landraces) in the landraces in agronomic group 2 (Tables 1 and 2). The agromorphological traits affected by the method of regeneration were ain and dmy. The mean values of six variables during the 2 years of evaluation are shown in Tables 1 and 2.

The five enzyme systems revealed the following polymorphic loci: PGI-2; PGM-1; ACP-1; PEX-1; SDH-1. The *L. temulentum* pattern was monomorphic and homozygous with the following fixed alleles: PGI-2a; PGM-1a; ACP-1d; PEX-1d; SDH-1b.

The allele frequencies in the five loci for the original and regenerated landraces are shown in Tables 3 and 4. Twenty-two alleles were identified in the original populations. PGI-2 was the most polymorphic locus, with up to seven alleles, while PGM-1 was the least polymorphic with up to three alleles. Out of 22 alleles, 15 can be considered as common and widespread, two as common and localized, four as rare and widespread, and one as rare and localized.

Twenty-two alleles were also identified in the regenerated landraces. PGI-2 was the most polymorphic locus, with up to seven alleles, while PGM-1 was the least polymorphic with up to three alleles. Out of 22 alleles, 15 can be considered as common and widespread, two as common and localized, two as rare and widespread and three as rare and localized.

Allele frequencies in the regenerated landraces differed significantly from the original landraces in four out of

Table 1. Mean values (SD), obtained in 2009, of six agronomic traits in the different landraces under study, compared with the commercial cultivar 'Vitesse'

	hed	dmy	alt	ain	flw	fll
Luarca	101.2b (1.5)	30.2c (10.4)	64.7b (2.3)	77.9c (7.5)	8.0 (0.2)	14.1b (0.7)
Luarcar	103.4b (1.3)	25.2c (9.2)	74.5b (2.1)	46.9d (6.6)	7.8 (0.3)	13.1b (0.7)
Pravia	99.8b (1.4)	32.5c (10.1)	62.2b (2.3)	79.1c (7.3)	7.2 (0.2)	13.4b (0.7)
Praviar	109.1b (1.3)	35.9c (8.8)	74.3b (2.0)	80.3c (6.4)	7.2 (0.3)	13.9b (0.7)
Prendes	101.9b (1.5)	25.4c (10.7)	61.4b (2.4)	76.7c (7.7)	7.7 (0.2)	15.8b (0.8)
Prendesr	103.3b (1.2)	33.3c (8.7)	82.2b (2.0)	65.1c (6.3)	7.7 (0.3)	14.2b (0.7)
Ordes	124.7a (1.3)	78.9b (9.4)	104.0a (2.1)	97.7b (6.8)	8.8 (0.2)	20.8a (0.7)
Ordesr	131.6a (1.2)	92.5a (8.6)	109.5a (1.9)	127.2a (6.2)	13.3 (0.3)	22.2a (0.6)
Padrón	128.4a (1.2)	79.7b (8.5)	106.7a (1.9)	112.7b (6.2)	8.9 (0.2)	21.7a (0.6)
Padrónr	123.8a (1.2)	65.8b (8.3)	104.8a (1.9)	105.9b (6.0)	8.2 (0.3)	18.7a (0.6)
'Vitesse'	134.4 (0.6)	90.9 (7.5)	115.4 (1.7)	140.5 (8.6)	8.5 (0.1)	25.1 (0.7)

hed, time of inflorescence emergence (number of days from 1 January); dmy, total annual dry matter yield (g dry matter yield per individual plant); alt, plant height (total height in cm); ain, number of inflorescences (number of flower spikes per individual plant); flw, maximum width of the flag leaf (mm); fll, maximum length of the flag leaf (cm).
Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$; Duncan's test).

https://doi.org/10.1017/S147926211100089X Published online by Cambridge University Press

Table 2. Mean values (SD), obtained in 2010, of six agronomic traits in the different landraces under study, compared with the commercial cultivar 'Vitesse'

	hed	dmy	alt	ain	flw	fll
Luarca	100.9b (1.2)	24.5d (8.3)	67.2b (1.8)	58.8c (5.7)	8.5 (0.2)	13.9b (0.6)
Luarcar	103.7b (1.3)	25.2d (8.5)	66.6b (1.9)	50.2c (6.1)	7.8 (0.3)	12.8b (0.6)
Pravia	103.9b (1.3)	35.7c (8.5)	72.9b (1.8)	86.9b (5.8)	7.5 (0.2)	14.2b (0.6)
Praviar	104.1b (1.4)	19.0d (9.2)	63.6b (2.1)	41.2c (6.6)	7.0 (0.3)	13.2b (0.7)
Prendes	100.1b (1.3)	29.6d (8.5)	70.5b (1.8)	60.2c (5.8)	8.4 (0.2)	14.7b (0.6)
Prendesr	103.0b (1.3)	24.3d (8.7)	68.3b (2.0)	53.3c (6.3)	8.2 (0.3)	13.7b (0.7)
Ordes	123.4a (1.4)	95.1b (9.8)	97.5a (2.1)	97.9a (6.7)	8.9 (0.2)	18.0a (0.7)
Ordesr	121.5a (1.3)	135.8a (8.7)	100.5a (2.0)	91.9a (6.3)	9.6 (0.3)	19.3a (0.7)
Padrón	128.3a (1.2)	75.9b (8.4)	99.3a (1.8)	103.4a (5.7)	8.4 (0.2)	16.9a (0.6)
Padrónr	129.7a (1.3)	73.0b (8.6)	97.9a (1.9)	106.1a (6.2)	7.9 (0.3)	15.5a (0.6)
'Vitesse'	139.7 (0.6)	118.9 (7.5)	111.0 (1.8)	154.7 (9.1)	7.8 (0.1)	16.7 (0.5)

hed, time of inflorescence emergence (number of days from 1 January); dmy, total annual dry matter yield (g dry matter yield per individual plant); alt, plant height (total height in cm); ain, number of inflorescences (number of flower spikes per individual plant); flw, maximum width of the flag leaf (mm); fll, maximum length of the flag leaf (cm).
Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$; Duncan's test).

15 instances (5 polymorphic loci × 3 landraces) in the landraces in agronomic group 1 (Table 3) and in four out of ten instances (5 polymorphic loci × 2 landraces) in the landraces in agronomic group 2 (Table 4). According to the classification used by Marshall and Brown (1975), loss of the following rare alleles (frequency <0.05) occurred: the rare widespread allele 'a' from the PGI-2 locus of the Padrón landrace; the rare localized allele 'c*' from locus PGI-2 present in the Pravia landrace; the rare localized allele 'd' from locus SDH-1 present in the Luarca landrace, but which was not present in the regenerated landrace. For the original landraces, the mean number of alleles per locus was 3.7, and 3.6 for the regenerated landraces (Tables 3 and 4). The average expected heterozygosity value varied across original populations, ranging from 0.373 to 0.482 with a mean value of 0.448. The highest value of He was obtained in the original Ordes landrace. For the regenerated landraces, He varied from 0.387 to 0.478 with a mean value of 0.447. The highest value of He was obtained in the regenerated Padrón landrace.

The highest fixation indices (F_{IS} and $F_{IT} > 0.1$) were obtained for PGM-1, ACP-1, PEX-1 and SDH-1, indicating the heterozygote deficits. The fixation indices were lower (F_{IS} and $F_{IT} < 0.1$) for locus PGI-2 than for the other loci, showing that no forces are acting within the populations for this locus. The average value of population differentiation (F_{ST}) was higher in the regenerated landraces ($F_{ST} = 0.034$) than in the original landraces ($F_{ST} = 0.021$), suggesting a higher genetic differentiation between them. The proportion of diversity between the landraces relative to total diversity can be expressed as $F_{ST}/(1 - F_{ST})$ (Nei, 1977). In this study, the total diversity in the original landraces was $0.021/0.979 = 0.021$, i.e. only 2.1% of the total genetic diversity for the five loci studied was due to differentiation among the landraces.

Discussion

All landraces were collected from their original locations, and a balanced seed sample of at least 50 plants was obtained. This was considered to yield a sample of seeds representative of the original panmictic population (Tyler *et al.*, 1984). According to Yonezawa *et al.* (1995), this technique is adequate for the detection of most of the genetic variability. However, there is a risk of the loss of some of this variability during the regeneration of accessions. Balfourier *et al.* (1994) observed a loss of rare alleles in *L. perenne* in a sample of 16–25 plants in a polycrossing trial, although the loss was compensated by a random increase in the frequency of these alleles in the other accessions within same group, producing a 'buffer effect'.

When a population is regenerated for seed, a decision must be reached with respect to the number of individuals to be regenerated, and it is recommended to use 100 or more plants in genetically heterogeneous accessions to avoid large losses of alleles and maximize seed yield (Sackville Hamilton *et al.*, 1998).

The classification of alleles according to their frequencies and distribution showed that most alleles (15) were common and widespread, indicating that they are likely to be captured, irrespective of the sampling strategy employed (Marshall and Brown, 1975). Marshall and Brown (1975) argued that the common localized alleles

https://doi.org/10.1017/S147926211100089X Published online by Cambridge University Press

Table 3. Allele frequencies at five loci in the landraces of agronomic group 1 (Luarca, Pravia and Prendes) and their respective regenerated landraces (Luarcar, Praviar and Prendesr), and population genetic statistics

		Landraces					
Locus	Allele	Luarca *N*	Luarcar *N*	Pravia *N*	Praviar *N*	Prendes *N*	Prendesr *N*
PGI-2		98	135	111	106	103	109
	a	0.000	0.000	0.009	0.009	0.000	0.000
	b	0.041	0.022	0.063	0.057	0.029	0.027
	c	0.102	0.081	0.072	0.095	0.067	0.092
	d	0.826	0.838	0.784	0.802	0.826	0.854
	e	0.031	0.059	0.054	0.028	0.078	0.027
	a +	0.000	0.000	0.009	0.009	0.000	0.000
	c*	0.000	0.000	0.009	0.000	0.000	0.000
	χ^2		7.83		4.44		3.87
PGM-1		99	139	111	105	103	86
	a	0.778	0.828	0.747	0.619	0.786	0.756
	b	0.222	0.172	0.235	0.333	0.214	0.244
	c	0.000	0.000	0.018	0.048	0.000	0.000
	χ^2		1.71		12.56**		0.33
ACP-1		99	133	114	106	102	109
	a	0.080	0.008	0.035	0.028	0.078	0.064
	b	0.667	0.639	0.667	0.623	0.667	0.578
	c	0.182	0.293	0.263	0.293	0.226	0.340
	d	0.071	0.060	0.035	0.056	0.029	0.018
	χ^2		17.89**		3.25		8.49*
PEX-1		100	102	113	96	100	113
	a	0.060	0.029	0.080	0.084	0.100	0.062
	b	0.560	0.500	0.345	0.427	0.570	0.433
	c	0.380	0.471	0.531	0.479	0.330	0.487
	d	0.000	0.000	0.044	0.010	0.100	0.018
	χ^2		3.44		6.00		16.34**
SDH-1		96	109	78	78	96	84
	a	0.031	0.055	0.051	0.077	0.083	0.107
	b	0.761	0.817	0.821	0.782	0.698	0.679
	c	0.198	0.128	0.115	0.128	0.219	0.214
	d	0.010	0.000	0.013	0.013	0.000	0.000
	χ^2		4.67		1.93		1.02
A		3.4 (0.4)	3.2 (0.4)	4.4 (0.7)	4.2 (0.5)	3.4 (0.4)	3.4 (0.4)
He		0.373 (0.06)	0.387 (0.05)	0.432 (0.05)	0.462 (0.05)	0.433 (0.05)	0.449 (0.06)

N, number of data; *A*, mean number of alleles per locus; He, Hardy–Weinberg expected heterozygosity (standard errors in parentheses).
The χ^2 value compares the proportion of alleles in the original populations with those of the regenerated populations.
Values were significantly different (*$P < 0.05$ and **$P < 0.01$).

(two alleles in original and regenerated landraces) merit priority in sampling, because these alleles confer adaptation to local conditions.

Lawrence *et al.* (1995) recommend that a population sample must maintain alleles at a frequency of at least 0.05. This type of allele, referred to as 'common' by Brown (1978), is considered adaptive and must be maintained in a population. Marshall and Brown (1975) argue that rare alleles are probably low in adaptative value and are of less interest to breeders. Loss of three rare alleles occurred with the regeneration method used.

According to Frankel *et al.* (1995), the maintenance of alleles (average number of alleles per locus) is more important than the maintenance of a precise allele frequency in the conservation of plant genetic resources for agriculture.

The regeneration method in which a balanced mix of seed was produced did not result in the loss of any common alleles. Johnson (1998) reached similar conclusions after comparing the genetic structure of three original populations of Italian ryegrass with the same populations regenerated by three different methods.

According to Brown (1978), both the mean heterozygosity and the number of alleles per locus represent key components of genetic diversity. A reduction in heterozygosity may suggest a loss in diversity and a

https://doi.org/10.1017/S147926211100089X Published online by Cambridge University Press

Table 4. Allele frequencies at five loci in the landraces of agronomic group 2 (Ordes and Padrón) and their respective regenerated landraces (Ordesr and Padrónr), and population genetic statistics

		Landraces			
Locus	Allele	Ordes *N*	Ordesr *N*	Padrón *N*	Padrónr *N*
PGI-2		140	140	142	143
	a	0.014	0.057	0.035	0.000
	b	0.157	0.186	0.190	0.147
	c	0.207	0.179	0.127	0.056
	d	0.594	0.543	0.606	0.741
	e	0.021	0.021	0.035	0.049
	a +	0.000	0.000	0.007	0.007
	c*	0.007	0.014	0.000	0.000
	χ^2		25.92**		12.31
PGM-1		145	153	142	119
	a	0.807	0.876	0.796	0.706
	b	0.193	0.124	0.204	0.294
	χ^2		4.25*		6.48*
ACP-1		111	136	143	123
	a	0.072	0.059	0.105	0.098
	b	0.649	0.699	0.692	0.691
	c	0.234	0.213	0.196	0.195
	d	0.045	0.029	0.007	0.016
	χ^2		1.53		0.45
PEX-1		107	148	142	147
	a	0.037	0.020	0.035	0.075
	b	0.383	0.345	0.359	0.367
	c	0.580	0.635	0.606	0.558
	χ^2		2.95		7.41*
SDH-1		112	123	110	113
	a	0.125	0.146	0.091	0.080
	b	0.679	0.683	0.655	0.637
	c	0.196	0.171	0.254	0.283
	χ^2		1.02		0.49
A		3.6 (0.7)	3.6 (0.7)	3.6 (0.7)	3.4 (0.5)
He		0.482 (0.05)	0.457 (0.07)	0.476 (0.04)	0.478 (0.02)

N, number of data; *A*, mean number of alleles per locus; He, Hardy–Weinberg expected heterozygosity (standard errors in parentheses).
The χ^2 value compares the proportion of alleles in the original populations with those of the regenerated landraces.
Values were significantly different (*$P < 0.05$ and **$P < 0.01$).

possible trend towards fixation (homozygosity) and loss of alleles at a given locus. In this study, no clear reduction in heterozygosity or in the number of alleles per locus was observed in the regenerated landraces, thus confirming the results obtained by Johnson (1998).

In this study, allele frequencies in the multiplied landraces deviated from those in the original landraces in 32% of direct comparisons. These results are consistent with those of Johnson (1998), who showed that there was a genetic shift in allele frequencies in several enzyme markers, as a result of regeneration of annual ryegrass accessions mainly with bulk seed samples. Other studies have shown considerable changes in allele frequencies after regeneration in *Brassica oleracea* accessions (Soengas *et al.*, *Secale cereale* (Chebotar *et al.*, 2003), but smaller2009) and changes in *Zea mays* (Reedy *et al.*, 1995) and *Triticum aestivum* (Börner *et al.*, 2000).

Several other factors may cause shifts in allele frequencies during regeneration (Sackville Hamilton and Chorlton, 1997), including the effects of genetic drift, selection and contamination with alien genes.

Genetic drift was probably the result of a reduced effective population size associated with high variation in seeds per plant (Heywood, 1986), and although a balanced sample was used with respect to weight of seeds, there was some variability in seed size per plant (data not shown) within accessions. Therefore,

https://doi.org/10.1017/S147926211100089X Published online by Cambridge University Press

seed samples of the same weight may have contained a different number of seeds so that maternal effects may have occurred. The effect of genetic drift can be reduced by keeping the effective population size to a minimum of 100 plants. To reach this objective, the same number of seeds per plant should be taken at harvest during regeneration.

Genetic shifts may have resulted from natural selection because the regeneration plots were not in the ecological region of origin of all landraces (Luarca, Prendes and Pravia landraces), and the regeneration environment may select some genotypes in preference to others in a population (Sackville Hamilton and Chorlton, 1997).

Although regeneration is carried out in isolated plots, some cases of contamination (<5%) may also take place, as indicated by Bradley and Johnson (1997) with modest isolation distances of between 22 and 27 m in annual ryegrass. Such effects would become more pronounced with numerous regeneration cycles.

The isozyme study revealed a deficit of heterozygotes at most of the loci, with the exception of PGI-2. According to Fearon *et al.* (1983), this is explained by the existence of a gametophytic self-incompatibility system of two multiallelic loci, S and Z. Both loci are linked to the PGI-2 locus on chromosome 6. The presence of a two-locus self-incompatibility system may inhibit fertilization in specific crosses where both S and Z alleles are shared by the parents and thus encourage heterozygosity (Cornish *et al.*, 1980a).

Heterozygote deficiencies in the present study may result from genetic drift, selection and subdivision of the population into separate breeding units, i.e. the Wahlund effect (Brown, 1978; Sproule and Dancik, 1996; Genlou and Salomon, 2003). Null alleles (lack of enzyme activity) may also contribute to the observed deficit (Cornish *et al.*, 1980b), but the frequency at which they occur remains uncertain.

Although the isozyme study provides interesting data on how the regeneration procedures affect the genetic structure of populations, the most important characters in plant genetic resources are agronomic and/or quantitative. Isozyme data cannot predict genetic changes in these characters. In this study, agromorphological characters in the regenerated landraces deviated from the original landraces in 20% of direct comparisons. Breese and Tyler (1981) observed a drift associated with regeneration towards earlier flowering and higher seed production per spike in the earlier flowering perennial ryegrass but no significant differences were observed for annual species (Breese, 1973).

The regeneration procedure used with five accessions of Italian ryegrass appeared to maintain genetic diversity (heterozygosity and allelic richness) of accessions in balanced seed samples, although significant changes in allele frequencies and agromorphological characters occurred and three rare alleles were lost.

Since regeneration has negative effects on the genetic integrity of the accessions and is laborious, it should be reduced to a minimum by placing much more emphasis on the efficiency of seed preservation systems.

Acknowledgements

The authors acknowledge financial support from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria de España (INIA), project RF2006-00012-C02 (2006–2009).

References

Balfourier F, Charmet G and Ravel C (1994) Conservation of allelic multiplicity and genotypic frequency by pooling wild populations of perennial ryegrass. *Heredity* 73: 386–396.

Börner A, Chebotar S and Korzum V (2000) Molecular characterization of the genetic integrity of wheat (*Triticum aestivum* L.) germplasm after long-term maintenance. *Theoretical and Applied Genetics* 100: 494–497 doi:10.1007/s001220050064.

Bradley VL and Johnson RC (1997) Conservation of grass collections at the Western Regional Plant Introduction Station. *Proceedings of the XVIII International Grassland Congress,* 8–19 June 1997, Winnipeg, Manitoba, Saskatoon, Saskatchewan, Canada. 1–36, pp. 1–35.

Breese EL (1973) Genetic architecture and adaptation in grasses with special reference to *Lolium*. *International Meeting on Quantitative Inheritance, Polymorphisms, Selection and Environment,* 2–4 October 1972, University of Bologna, Bologna, Italy, pp. 73–81.

Breese EL (1989) *Regeneration and Multiplication of Germplasm Resources in Seed Genebanks: the Scientific Background*. Rome: International Board for Plant Genetic Resources, p. 69.

Breese EL and Tyler BF (1981) Regeneration of germplasm collections of forage grasses and legumes. In: Porceddu E, Jenquins G and Bulkema AA (eds) *Seed Regeneration in Cross-pollinated Species*. Rotterdam: Balkema, pp. 45–67.

Brown AHD (1978) Isozymes, plant population genetic structure and genetic conservation. *Theoretical and Applied Genetics* 52: 145–157.

Chebotar S, Röder MS, Korzum V, Saal B, Weber WE and Börner A (2003) Molecular studies on genetic integrity of open pollinating species rye (*Secale cereale* L.) after long-term genebank maintenance. *Theoretical and Applied Genetics* 107: 1469–1476 doi:10.1007/s00122-003-1366-1.

Cornish MA, Hayward MD and Lawrence MJ (1980*a*) Self-incompatibility in ryegrass. 4. Seed set in diploid *Lolium perenne* L. *Heredity* 44: 333–340.

Cornish MA, Hayward MD and Lawrence MJ (1980*b*) Self-incompatibility in ryegrass. 3. The joint segregation of S and PGI-2 in *Lolium perenne* L. *Heredity* 44: 55–62.

Fearon CH, Hayward MD and Lawrence MJ (1983) Self incompatibility in ryegrass. V. Genetic control in diploid *Lolium multiflorum*. *Heredity* 50: 35–45.

Frankel OH, Brown AHD and Burdon JJ (1995) The conservation of cultivated plants. In: Frankel OH, Brown AHD and Burdon JJ (eds) *The Conservation of Plant Biodiversity*. Cambridge: Cambridge University Press, pp. 79–117.

Genlou S and Salomon B (2003) Microsatellite variability and heterozygote deficiency in the arctic–alpine Alaskan wheatgrass (*Elymus alaskanus*) complex. *Genome* 46: 729–737.

Guy P, Ghesquière M, Charmet G and Prosperi JM (1989) Pooling accessions: advantages and disadvantages. *Report of a Working Group on Forages, (Third Meeting),* 9–12 January 1989, Mauguio, Montpellier, France. Rome: ECP/GR/IBPGR, pp. 35–49.

Hartl DL and Clark AG (1997) *Principles of Population Genetics*. 3rd edn. Sunderland, MA: Sinauer Associates, Inc., p. 542.

Hayward MD, Degennars GH, Balfourier F and Eickmeyer F (1995) Isozyme procedures for the characterisation of germplasm, exemplified by the collection of *Lolium perenne* L. *Genetic Resources and Crop Evolution* 42: 327–337.

Heywood JS (1986) The effect of plant size variation on genetic drift in populations of annuals. *American Naturalist* 27: 851–861.

Johnson RC (1998) Genetic structure of regeneration populations of annual ryegrass. *Crop Science* 38: 851–857.

Johnson RC, Bradley VI and Knowles RP (1996) Genetic contamination by windborne pollen in germplasm-regeneration plots of smooth bromegrass. *Plant Genetic Resources Newsletter* 106: 30–34.

Lawrence MJ, Marshall DF and Davies P (1995) Genetics of conservation. I. Sample size when collecting germplasm. *Euphytica* 84: 89–99.

Lee LS and Henry RJ (2001) Commercial applications of plant genotyping. In: Henry RJ (ed.) *Plant Genotyping: The DNA Fingerprinting of Plants*. Wallingford: CAB International, pp. 265–273.

Lloveras J (1987) Forage production and quality of several crop rotations and pastures in northwestern Spain. *Grass and Forage Science* 42: 241–247.

López JE, González E, Castro J and Oliveira JA (2011) Colección de especies pratenses para la España Húmeda. *Agricultura* 938: 168–172.

Marshall DR and Brown ADH (1975) Optimum sampling strategies in genetic conservation. In: Frankel OH and Hawkes JG (eds) *Crop Genetic Resources for Today And Tomorrow*. Cambridge: Cambridge University Press, pp. 53–80.

Medina RD, Faloci MM, Marassi MA and Mroginski LA (2005) Identificación de variedades de arroz (*Oryza sativa* L.) cultivadas en Argentina mediante marcadores bioquímicos: su utilidad potencial para el registro de cultivares. *Plant Genetic Resources Newsletter* 143: 1–7.

Nei M (1977) *F*-statistics and analysis of gene diversity in subdivided populations. *Annals of Human Genetics* 41: 225–233.

Oliveira JA, Lindner R, Bregu R, García A and González A (1997) Genetic diversity of westerworld ryegrass landraces in northwest Spain. *Genetic Resources and Crop Evolution* 44: 479–487.

Piñeiro J and Pérez M (1986) El interés agronómico de ecotipos españoles de plantas pratenses. *Pastos* 44: 103–118.

Piñeiro J and Pérez M (1992) Mezclas pratenses de la España Húmeda. Ministerio de Agricultura, Pesca y Alimentación. *Hoja Divulgadora* Número 8/92 HD: 1–48.

Reedy ME, Knapp AD and Lamkey KR (1995) Isozyme allelic frequency changes following maize (*Zea mays* L.) germplasm regeneration. *Maydica* 40: 269–273.

Rodríguez NN, Fuentes JL, Coto O, Fuentes V, Ramírez IM, Becker D, Rodríguez I, González C, Xonia X, Román MI, Velásquez B and Rohde W (2009) Agro-morphologic traits, isoenzyme and DNA markers for estimating the polymorphism levels, discriminating capacity and informativeness in Avocado. *Revista CENIC Ciencias Biológicas* 40: 63–74.

Sackville Hamilton NR and Chorlton KH (1997) Regeneration of accessions in seed collections: a decision guide. *IPGRI/FAO Handbooks for Genebanks 5*. Rome: IPGRI.

Sackville Hamilton NR, Chorlton KH and Thomas ID (1998) Guidelines for the regeneration of accessions in seed collections of the main perennial forage grasses and legumes of temperate grasslands. In: Maggioni L, Marum P, Sackville Hamilton R, Thomas I, Gass T and Lipman E (eds) *Report of a Working Group on Forages, Sixth Meeting,* 6–8 March 1997, Beitostolen, Norway. Rome: IPGRI.

SAS Institute (1999) SAS/STAT user's guide, version 8. *SAS Technical Report*. Carry, NC: SAS Institute, Inc.

Snedecor GW and Cochran WG (1967) Statistical Methods. 6th edn. Ames, IA: The Iowa State Univesity Press, p. 593.

Soengas P, Cartea E, Lema M and Velasco P (2009) Effect of regeneration procedures on the genetic integrity of *Brassica oleracea* accessions. *Molecular Breeding* 23: 389–395.

Sproule AT and Dancik BP (1996) The mating system of black spruce in north-central Alberta, Canada. *Silvae Genetica* 45: 159–164.

Swofford DL and Selander RB (1981) BIOSYS 1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity* 72: 281–283.

Tyler BF, Chorlton KH and Thomas ID (1984) Characterization of collected *Lolium perenne* populations. *Report of the Welsh Plant Breeding Station,* 1983, Aberysthwyth, UK, pp. 29–32.

Virk PS, Zhu J, Newbury HJ, Bryan GJ, Jackson MT and Ford-Lloyd BV (2000) Effectiveness of different classes of molecular marker for classifying and revealing variation in rice (*Oryza sativa* L.) germplasm. *Euphytica* 112: 275–284.

Wright S (1965) The interpretation of population structure by *F*-statistics with special regards to systems of mating. *Evolution* 19: 395–420.

Yonezawa K, Nomura T and Morishima H (1995) Sampling strategies for use in stratified germplasm collections. In: Hodgkin T, Brown AHD, Van Hintum TJL and Morales EAV (eds) *Core Collections of Plant Genetic Resources*. New York: John Wiley and Sons, pp. 35–54.